

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>5</sup> :</b> <b>A61K 31/395</b>	<b>A2</b>	<b>(11) International Publication Number:</b> <b>WO 94/08578</b> <b>(43) International Publication Date:</b> 28 April 1994 (28.04.94)
<b>(21) International Application Number:</b> PCT/US93/09858 <b>(22) International Filing Date:</b> 14 October 1993 (14.10.93)  <b>(30) Priority data:</b> 07/961,250                      14 October 1992 (14.10.92)      US  <b>(71) Applicant:</b> THE GOVERNMENT OF THE UNITED STATES OF AMERICA, as represented by THE SECRETARY OF THE DEPARTMENT OF HEALTH AND HUMAN SERVICES [US/US]; Box OTT, Bethesda, MD 20892 (US).  <b>(72) Inventors:</b> WHITESELL, Luke ; 6006 Kingsford Road, Bethesda, MD 20817 (US). NECKERS, Leonard ; TREP-EL, Jane ; 5121 Wissioming Road, Bethesda, MD 20816 (US). MYERS, Charles ; 11111 Ralston Road, Rockville, MD 20852 (US).		<b>(74) Agents:</b> WEBER, Kenneth, A. et al.; Townsend and Townsend Kourie and Crew, Steuart Street Tower, 20th floor, One Market Plaza, San Francisco, CA 94105 (US).  <b>(81) Designated States:</b> AU, CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> <i>Without international search report and to be republished upon receipt of that report.</i>
<b>(54) Title:</b> TUMORICIDAL ACTIVITY OF BENZOQUINONOID ANSAMYCINS AGAINST PROSTATE CANCER AND PRIMITIVE NEURAL MALIGNANCIES  <b>(57) Abstract</b>  The invention provides methods and compositions for treating selected human malignancies including use of certain ansamycin benzoquinones.		

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FR	France	MR	Mauritania
AU	Australia	GA	Gabon	MW	Malawi
BB	Barbados	GB	United Kingdom	NE	Niger
BE	Belgium	GN	Guinea	NL	Netherlands
BF	Burkina Faso	GR	Greece	NO	Norway
BG	Bulgaria	HU	Hungary	NZ	New Zealand
BJ	Benin	IE	Ireland	PL	Poland
BR	Brazil	IT	Italy	PT	Portugal
BY	Belarus	JP	Japan	RO	Romania
CA	Canada	KP	Democratic People's Republic of Korea	RU	Russian Federation
CF	Central African Republic	KR	Republic of Korea	SD	Sudan
CG	Congo	KZ	Kazakhstan	SE	Sweden
CH	Switzerland	LI	Liechtenstein	SI	Slovenia
CI	Côte d'Ivoire	LK	Sri Lanka	SK	Slovak Republic
CM	Cameroon	LU	Luxembourg	SN	Senegal
CN	China	LV	Latvia	TD	Chad
CS	Czechoslovakia	MC	Monaco	TG	Togo
CZ	Czech Republic	MG	Madagascar	UA	Ukraine
DE	Germany	ML	Mali	US	United States of America
DK	Denmark	MN	Mongolia	UZ	Uzbekistan
ES	Spain			VN	Viet Nam
FI	Finland				

5                   TUMORICIDAL ACTIVITY OF BENZOQUINONOID ANSAMYCINS  
                  AGAINST PROSTATE CANCER AND PRIMITIVE NEURAL MALIGNANCIES

                  BACKGROUND OF THE INVENTION

                  Benzoquinonoid ansamycin antibiotics were isolated  
in the late 1970s from the culture broths of several  
10 actinomycete species. Their unusual ansa bridge structure  
generated considerable interest, and a number of compounds  
including herbimycin A (HA) and geldanamycin (GA) were  
screened as possible anti-retroviral and anti-tumor agents.  
Results against the usual test cell lines of the time such as  
15 L1210 leukemia and P-388 were disappointing. The  
concentrations required for anti-tumor activity were quite  
toxic in the whole animal and further development was  
abandoned.

                  A new wave of enthusiasm occurred in the late 1980s  
20 when it was noted that HA was able to revert the phenotype of  
tyrosine kinase oncogene-transformed cell lines at quite  
modest concentrations. Inhibition of angiogenesis and  
induction of differentiation in a number of model systems were  
also reported. HA and GA have been shown to induce  
25 differentiation in a number of *in vitro* model systems,  
reportedly due to their inhibition of src-family protein  
tyrosine kinases.

                  Pediatric and adult cancers of primitive neural  
derivation, whether metastatic, locally disseminated, or  
30 recurrent, are among those malignancies most refractory to  
cure by current multi-modality treatment regimens. New  
approaches to treatment are clearly needed.

                  SUMMARY OF THE INVENTION

35                   The invention provides a method for treating human  
malignancies selected from the group comprising primitive  
neuroectodermal tumors, prostate cancer, melanoma, and  
metastatic Ewing's sarcoma. The method includes administering

an effective dosage of an ansamycin benzoquinone to an animal which has the malignancy. The administration is preferably parenteral, such as intravenous.

5 The effective dosage is usually selected from a range of about 0.1 milligram of drug per kilogram body weight of the recipient animal (mg/kg) to about 20 mg/kg. Preferably, the effective dosage is selected from a range of about 1 mg/kg to about 10 mg/kg. More preferably, the effective dosage is about 5 mg/kg.

10 The ansamycin benzoquinone is typically selected from a group consisting of geldanamycin and its derivatives, herbimycin A and its derivatives, and macbecin I and its derivatives including macbecin II. The general chemical formula for the ansamycin benzoquinones is displayed in  
15 Figure 1. As noted, the side groups  $R_1$ ,  $R_2$ ,  $R_3$  and  $R_4$  are typically a hydrogen, a hydroxyl, an alkyl or an alkoxy. The term "alkyl" refers to substituents that are saturated hydrocarbon radicals. The alkyl groups may be straight-chain or branched-chain, limited only by steric hindrance. Shorter  
20 alkyl groups, such as 1-4 carbon atoms are preferred. The term "alkoxy" is used to refer to an alkyl radical which also bears an oxygen substituent that is capable of covalent attachment to another hydrocarbon radical (for example, a methoxy or ethoxy group). As with alkyl groups, shorter  
25 alkoxy groups are preferred. The term "independently selected" is used to indicate that the various R groups,  $R_1$  to  $R_4$ , may be identical or different.

The invention includes a method for treating human malignancies selected from the group comprising primitive  
30 neuroectodermal tumors, prostate cancer, melanoma, and metastatic Ewing's sarcoma comprising parenterally administering to a human an effective dosage of an ansamycin benzoquinone selected from the group consisting of geldanamycin and its derivatives, herbimycin A and its  
35 derivatives, macbecin I and its derivatives including macbecin II. The effective dosage is an amount sufficient to ameliorate symptoms or signs of the cancer. The amount is generally a range of from about 0.1 mg/kg to about 20 mg/kg.

The geldanamycin derivatives are preferably those in the group consisting of 17-des-O-methylgeldanamycin, geldanamycin acetate, 7',8'-benzodemethoxygeldanoxazone, hydrogeldanamycin-18,21-diacetate, 7'-(and 8')-  
5 fluorodemethoxygeldanazine, 7'-bromodemethoxygeldanoxazinone, 19-piperidinogeldanamycin, geldampicin, 8'-bromodemethoxygeldanoxazinone, 7'(or 8')-fluorodemethoxygeldanazine, and 17-amino-17-demethoxygeldanamycin.

10 The invention further includes a pharmaceutical composition having at least one pharmaceutically acceptable excipient and an amount of an ansamycin benzoquinone effective to treat cancer in a mammal to whom at least one dose of the composition is administered. The cancer is selected from a  
15 group including primitive neuroectodermal tumors, prostate cancer, melanoma, and metastatic Ewing's sarcoma.

Typically, the mammal is a human and the amount of ansamycin benzoquinone is selected from a range of from about 0.1 mg/kg to about 20 mg/kg. Preferably, the amount of  
20 ansamycin benzoquinone is selected from a range of from about 1 mg/kg to about 10 mg/kg. More preferably, the amount of ansamycin benzoquinone is about 5 mg/kg. The composition is preferably adapted for parenteral administration such as intravenous administration.

25 The ansamycin benzoquinone component of the composition is usually selected from a group consisting of geldanamycin and its derivatives, herbimycin A and its derivatives, macbecin I and its derivatives including macbecin II. The geldanamycin derivative is preferably selected from  
30 the group specified above.

#### BRIEF DESCRIPTION OF THE FIGURES

Fig. 1 is the chemical structure of ansamycin benzoquinones.

35 Fig. 2 graphically shows that benzoquinonoid ansamycins inhibit cell proliferation and survival in a dose dependent fashion.

Fig. 3 is a photograph of a DNA electrophoresis assay showing that cycloheximide inhibits HA-induced DNA degradation.

Fig. 4 demonstrates lack of benzoquinonoid ansamycin toxicity to primary neurons in culture.

Fig. 5 shows HA inhibition of prostate tumor growth in nude mice.

Fig. 6 shows that systemic treatment with HA of tumor-bearing mice inhibits tumor growth.

#### DESCRIPTION OF THE PREFERRED EMBODIMENT

Metastatic and locally disseminated cancers of primitive neural derivation remain among the most refractory to treatment. The invention shows that benzoquinonoid ansamycins possess very potent cytotoxic activity *in vitro* against a select range of human tumor lines and *in vivo* in a mouse model of certain human tumors. The invention also found little toxicity on primary neuron cultures, several hematopoietic cell lines and a mouse fibroblast line. Cell lines of the pediatric cancers neuroblastoma, neuroepithelioma and medulloblastoma were used as model systems in which to explore the therapeutic potential of ansamycin benzoquinones.

The benzoquinonoid ansamycins are potent cytotoxins *in vitro* against a panel of highly malignant human tumor cell lines possessing primitive neural features. Proliferation and/or survival of fibroblasts, primary neuronal cultures and several leukemia cell lines are unaffected at concentrations resulting in greater than 99% cell loss in sensitive lines. The tumorigenicity in nude mice of sensitive cell lines can also be markedly reduced by either systemic or topical administration of these agents without apparent toxicity to the whole animal. The cytotoxic action of these ansamycins is initiated very rapidly, is irreversible and clearly distinct from the delayed inhibition of *src*-family kinases that has been previously reported. Due to their potency, relative selectivity and novel mechanism(s) of action, these drugs can prove clinically useful in the therapy of a number of human cancers, particularly those of neural derivation.

The benzoquinonoid ansamycins are available commercially, or they can be isolated from natural sources. For example, production of geldanamycin is described by DeBoer, et al. "Geldanamycin, A New Antibiotic," *The Journal of Antibiotics* 23:9, 442-447 (September 1970). For isolation of herbimycin, see Omura, et al. "Herbimycin, A New Antibiotic Produced By A Strain Of *Streptomyces*," *The Journal of Antibiotics* 32:4, 255-261 (April 1979). For isolation of macbecins I and II, see Muroi, et al. "Macbecins I and II, New Antitumor Antibiotics," *The Journal of Antibiotics* 33:2, 205-212 (February 1980). Usually the parent compound, such as HA and GA, is isolated from fermentation broth and derivatives are produced by chemical manipulation of the parent. Preparation of derivatives involves conventional chemical techniques and is within the skill of the ordinary artisan. The ansamycin benzoquinones, such as HA and GA, are available from the National Cancer Institute (NCI) Repository for investigational use. They are commercially available from Bethesda Research Lab (BRL) in Rockville, Maryland.

When referring to treating a cancer, improved or decreased symptoms are included. That is, "treatment" is not limited to an objective regression of tumor size, but also includes the patient's report of his subjective status.

An effective amount of the compound is that amount which provides subjective relief of a symptom, decrease in the tumor burden, or decrease in an identifiable tumor marker. Administration of the compound is by any medically or pharmaceutically accepted route. Typically, the parenteral route is preferred. Examples include intravenous, intramuscular, and subcutaneous administration. Topical application is also effective. The dosing range is preferably from about 0.1 to about 20 mg/kg/day based on the patient's body weight. More specifically, the range is from about 1 to about 10 mg/kg/day.

The dosage of the benzoquinonoid ansamycin depends on many factors that are well known to those skilled in the art. The factors include, for example, the route of administration, the potency of the particular compound, the

condition being treated, and the patient's age, weight, and general state of health including cardiac, hepatic and renal function.

5 A preferred dosing schedule is a parenteral or topical dose usually once or twice a day for a limited period, typically about 5 to 10 days. The schedule is preferably repeated at a suitable interval, usually at about 14 to 28 days with the first day of the first cycle counted as day one. Alternatively, the dose can be given every other day or every 10 three to four days for a total of about three to ten doses. A preferred parenteral route is intravenous (IV). Preferably, the IV dose is infused over about 30 to 60 minutes.

Compositions of the present invention are presented for administration to humans and animals in unit dosage forms, 15 such as tablets, capsules, pills, powders, granules, aqueous solutions or suspensions and water-in-oil emulsions containing suitable quantities or formulations of a benzoquinonoid ansamycin.

The term "unit dosage form" refers to physically 20 discrete units suitable as unitary dosages for human subjects and animals, each unit containing a predetermined quantity of active material calculated to produce the desired pharmaceutical effect in association with the required pharmaceutical diluent, carrier or vehicle. Some examples of 25 suitable unit dosage forms are tablets, capsules, pills, powder packets, wafers, granules, teaspoonfuls, tablespoonfuls, droppersful, ampoules, and vials.

The benzoquinonoid ansamycin may be combined or mixed with various solutions and other compounds as is known 30 in the art. For example, it may be administered in water, saline or buffered vehicles. The benzoquinonoid ansamycin may be administered by any conventional method including oral, topical and parenteral (e.g., intravenous or intramuscular) injection. The treatment may consist of a single dose or a 35 plurality of doses over a period of time. The drugs can be combined with appropriate doses of compounds. A pharmaceutically effective amount of the drugs can be employed

with a pharmaceutically acceptable carrier such as an additive or diluent.

A preferred formulation for parenteral use is an emulsion including the ansamycin benzoquinone and at least one emulsifying agent. For example, the vehicle Cremophor can be used as an emulsifier. Emulsifiers which are nonionic and which contain complex fatty acids are preferred. Additionally, detergents may be useful in preparing the emulsion. Another preferred formulation includes modifying the parent compound, such as HA or GA, to a salt which would be soluble in an aqueous solution. For example, esterification of reactive groups can be carried out to produce water soluble homologues or analogues of the ansamycin benzoquinone. Because the parent compounds tend to be insoluble in water, administration as an aqueous solution without modification of the parent is a lesser preferred, although a feasible, alternative.

#### EXAMPLES.

##### EXAMPLE 1.

Cell Cultures. CHP-100 cells were obtained from Dr. A. Evans (Children's Hospital of Philadelphia). The cell lines TC-32 and NIH3T3 were obtained from Dr. M. Tsokos (NCI, Laboratory of Pathology). All other cell lines used were purchased from the American Type Culture Collection ATCC, Rockville, MD). Primary neonatal rat cortical neurons were established using standard techniques and supplied by Dr. M. Koenig (Armed Forces Research Institute, Washington, DC). All cell lines were tested using a Mycotect<sup>R</sup> kit (GIBCO Laboratories, Grand Island, NY) and found free of mycoplasma contamination. All culture media were supplemented with 10% fetal bovine serum (FBS) (Whittaker Bioproducts, Walkersville, MD).

The cell lines CHP-100, TC-32, IMR-32, SKNSH, CEM and HL-60 were grown in RPMI 1640 (Biofluids, Inc., Rockville, MD). The cell lines D283 Med, D341 Med, SKNMC, SK-MEL-1, SK-MEL-2, and RPMI 7951 were cultured in Eagle's MEM with 1% non-essential amino acids and 1mM sodium pyruvate (all from

Biofluids, Inc). NIH3T3 cells were cultured in Dulbecco's MEM with 4.5 g/L glucose (Biofluids, Inc). Herbimycin A (National Service Center NSC 305978) and geldanamycin (NSC 122750) were obtained from the Drug Synthesis and Chemistry Branch,  
5 National Cancer Institute (NCI), formulated as 2mg/ml stock solutions in dimethyl sulfoxide (Sigma Chemical Co., St. Louis, MO) and stored at 4°C in the dark.

Selective Cytotoxic Activity of HA and GA Against Primitive Neural Cell Lines. Data are graphed in Fig. 2 using  
10 the following legend. Open circles refer to the cell line CHP-100. Closed boxes refer to the cell line NIH 3T3. Panel A had HA added to the culture medium and <sup>3</sup>H-thymidine incorporation was assayed 48 hours later. Panel B had GA added to the culture medium and <sup>3</sup>H-thymidine incorporation was  
15 assayed 48 hours later. Panel C had HA added to the culture medium and MTT reduction was assayed 5 days later. Panel D had GA added to the culture medium and MTT reduction was assayed 5 days later. Data are expressed as percent cpm or optical density relative to control wells plated  
20 simultaneously without the addition of the drug. All points represent the mean of triplicate determinations. Standard deviations are less than 10%.

Figure 2, panel A demonstrates potent growth inhibitory activity of HA against the primitive  
25 neuroectodermal cell line CHP-100 as measured by inhibition of <sup>3</sup>H-thymidine incorporation after two days of culture with the drug. This cell line is approximately 10-fold more sensitive *in vitro* to the inhibitory activity of HA than the non-tumorigenic mouse fibroblast cell line NIH3T3. Panel B  
30 demonstrates a similar pattern of cell type selectivity for the related ansamycin GA, and GA is approximately ten-fold more potent than HA.

Experiments were also carried out with these two cell lines assessing the effects of HA and GA on survival  
35 rather than inhibition of DNA synthesis. Automated analysis of MTT reduction by treated cells in a 96 well microtiter format allowed generation of dose response curves similar to panels A and B, but the endpoint was the relative number of

viable cells remaining after 5 days in culture. As panels C and D demonstrate, concentrations of HA or GA that result in complete cell loss with CHP-100 do not affect the fibroblast cell line. Relative potency and selectivity were similar by MTT analysis and thymidine incorporation analysis. The effect seen in MTT experiments was not simple inhibition of growth. Values actually declined from the time of initial plating, indicating cell loss (data not shown). Microscopic examination of treated wells prior to analysis also confirmed that only cellular debris remained.

Cell Proliferation and Survival Studies. Thymidine incorporation studies were performed as previously described (Whitesell L, Rosolen A, Neckers LM, "Episome-generated N-myc antisense RNA restricts the differentiation potential of primitive neuroectodermal cell lines", *Mol. Cell. Biol.* 11: 1360-1371 (1991)) except that serial dilutions of HA or GA were added to triplicate wells in 100  $\mu$ L of the appropriate growth medium for the cell line being tested.

Plates were cultured for 48 to 72 hours and then pulsed with [methyl-<sup>3</sup>H]-thymidine (Dupont, Boston, MA) for 4 hours, followed by automated cell harvest and liquid scintillation counting.

As an assay of relative viable cell number, mitochondrial reduction of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was used (Alley MC, et al., "Feasibility of drug screening with panels of human tumor cell lines using a microculture tetrazolium assay", *Cancer Res.*, 48: 589-601 (1988)). Ninety-six well tissue culture plates were set-up as described by Alley, but cultured for 5 to 6 days. 20  $\mu$ L of a 5 mg/ml solution of MTT (Sigma, St. Louis, MO) in phosphate buffered saline (PBS) was added to all wells and plates were incubated for 4 more hours in darkness.

Plates were centrifuged, medium removed and 150  $\mu$ L of dimethyl sulfoxide (Sigma, St. Louis, MO) added to each well. After a 10 minute incubation in darkness with shaking, the optical density at 540 nm was determined using a Biokinetics plate reader (Model EL-312, Bio-Tek Instruments

Inc., Winooski, VT). Optical density was used as a measure of the formazan concentration generated in each well.

#### EXAMPLE 2.

5                   Characteristics of HA and GA cytotoxicity. Having demonstrated that HA and GA are actually cytotoxic to CHP100 cells, characteristics of this cytotoxicity was examined. Cell death can be an active or passive process depending on cell type and initiating stimulus. DNA degradation is often  
10 used as an endpoint for both processes. A requirement for ongoing protein synthesis in the transduction of cell death suggests an active type of program.

CHP-100 cells ( $5 \times 10^6$ /10 cm tissue culture dish) were plated in the presence of HA (500 nM), 80 nM cycloheximide (a  
15 concentration previously determined to inhibit protein synthesis by 75% as measured by  $^3\text{H}$ -leucine incorporation) or a combination of the two. Sixty-eight hours after plating, the cells were harvested by trypsinization and cell number and viability determined by trypan blue exclusion in a  
20 hemocytometer chamber. The cells were washed once in PBS and high molecular weight DNA prepared according to a previously reported method (Rodriguez-Tarduchy G, Lopez-Rivas A., "Phorbol esters inhibit apoptosis in IL-2 dependent T-lymphocytes", *Biochem. Biophys. Res. Comm.*, 164: 1069-1075  
25 (1989)).

Briefly, cell pellets were incubated at  $37^\circ\text{C}$  for at least 4 hours in 0.5mL of lysis buffer (200mM TRIS pH8.5, 100 mM EDTA, 50  $\mu\text{g}/\text{ml}$  proteinase K, 1% SDS). The DNA was phenol extracted and the aqueous phase dialyzed overnight against 10  
30 mM TRIS pH7.5, 1mM EDTA. The DNA was incubated at  $37^\circ\text{C}$  with 50  $\mu\text{g}/\text{mL}$  RNase A. After 5 hours, 120  $\mu\text{g}/\text{mL}$  proteinase K was added and the incubation continued for 5 more hours. DNA was extracted with phenol followed by chloroform and precipitated with ethanol/sodium acetate. After re-dissolving in water, 5  
35  $\mu\text{g}$  of DNA was loaded per lane on a 1.4% agarose gel and electrophoresed for 16 hours at 35 volts prior to staining with ethidium bromide and photography.

The lanes in Fig. 3 have the following meaning. CTRL indicates a control and no drugs were added. HA means that 500 nM HA were added. CHX means that 80 nM cycloheximide were added. CHX/HA means both drugs were added simultaneously. DNA was isolated and electrophoresed as described above. Degradation of high molecular weight DNA is apparent only in lane HA. Figure 3 demonstrates that protein synthesis is necessary for HA-induced cell death. In this experiment, cells were cultured in the presence of 500 nM HA with or without the non-specific- protein synthesis inhibitor cycloheximide. After 68 hours, cells were harvested, assayed for viability and high molecular weight DNA was prepared. Viability of untreated cells was 82%, that of 80 nM cycloheximide-treated cells was 57%, that of HA-treated cells 26% and that of HA/cycloheximide-treated cells was 66%. Cycloheximide treatment clearly inhibited the cytotoxic activity of HA.

Analysis of high molecular weight DNA revealed substantial DNA degradation in the HA-treated cells (Figure 3, lane HA), but the nucleosomal cleavage characteristic of apoptosis was not seen. However, DNA degradation was abrogated by co-incubation of the cells with cycloheximide (lane CHX/HA). The quality of high molecular weight DNA in that lane is indistinguishable from that of either untreated (lane CTRL) or cycloheximide alone-treated cells (lane CHX). These findings confirm that HA is cytotoxic to sensitive cells and that this cytotoxicity requires active cellular participation.

To further evaluate the spectrum of activity displayed by HA and GA, a panel of primitive neural cell lines was screened using both inhibition of <sup>3</sup>H-thymidine incorporation and reduction of MTT as endpoints. See Table 1 which is a summary of dose response data obtained by thymidine assay for a variety of both neural and non-neural cell lines. Non-neural cell types such as the hematopoietic lines HL-60 and CEM were quite resistant to growth inhibition by HA and GA. All primitive neuroectodermal cell lines examined, both peripheral nervous system-derived (e.g. CHP-100, SKNMC, TC-32)

and central nervous system-derived (e.g. D283 Med, D341 Med) have proven quite sensitive to HA and GA. Cells from a primary culture of the malignant pleural effusion of a patient with relapsed, heavily pre-treated Ewing's sarcoma were also sensitive to HA as determined by MTT assay ( $IC_{95} = 400$  nM).

Cells of more mature neural phenotype were relatively insensitive to the cytotoxic activity of HA. Thymidine incorporation data for the neuroblastoma cell lines IMR-32 and SKNSH are shown in Table 1. The highly differentiated rat pheochromocytoma cell line PC-12 was also relatively insensitive to HA by MTT assay ( $IC_{95} > 500$  nM).

Data are presented as bar graphs in Fig. 4. HA at 472 nM or GA at 40 nM was added to established neonatal rat cerebellar neuron cultures. Plates were incubated for a further 4 days and reduction of MTT dye reflecting viable cell number per well was then assayed as optical density at 540 nm. The height of the bars represents the mean absorbance of quadruplicate wells. Error bars indicate the standard deviation of the mean.

Figure 4 demonstrates that primary cultures of neonatal rat cerebellar neurons were not affected by culture in 472 nM HA or 40 nM GA. This experiment was also repeated several times with neonatal rat cerebral cortical neurons with the same results. Lastly, the melanoma cell lines SK-MEL-1, SK-MEL-2 and RPMI 7951 were also screened against HA by MTT assay. The one line found to be sensitive, RPMI 7951 (HA  $IC_{50} = 125$  nM) is reported to possess the most primitive, least differentiated phenotype as defined by Houghton et al., "Surface antigens of melanocytes and melanomas", *J. Exp. Med.*, 156:1755-1766 (1982).

TABLE 1

*Selectivity of growth inhibition by HA and GA*

Data are derived from dose response curves generated by quantification of <sup>3</sup>H-thymidine incorporation by indicated cell lines 48 hours after plating in various concentrations of the indicated drug. Cell lines are grouped in decreasing order of sensitivity

10	Cell Line	Drug		Geldanamycin (nM)
		Herbimycin A (nM)		
		IC <sub>50</sub> <sup>a</sup>	IC <sub>95</sub> <sup>b</sup>	IC <sub>50</sub>
				IC <sub>95</sub>
	CHP-100	62	236	5
	TC-32	31	236	5
	SKNMC	62	236	8
				25
15	D283 Med	62	236	N.D. <sup>c</sup>
	D341 Med	10	30	N.D.
	IMR-32	236	>472	N.D.
	SKNSH	236	>472	N.D.
20	NIH3T3	710	3780	80
	HL-60	>472	>472	>40
	CEM	>472	>472	>40

<sup>a</sup> IC<sub>50</sub> = Concentration of drug resulting in 50% decrease in mean cpm of triplicate wells relative to untreated control wells.

<sup>b</sup> IC<sub>95</sub> = Concentration of drug resulting in a 95% decrement.

<sup>c</sup> N.D. = evaluation not done.

**EXAMPLE 3.**

Tumorigenicity Studies. CHP-100 cells were harvested from subconfluent monolayers and resuspended at 5x10<sup>7</sup> cells/mL in PBS. 100 μL were inoculated subcutaneously in the right and left inguinal areas of six week old male athymic nude mice (Frederick Cancer Research Facility, Frederick, MD) on day 0.

Therapy with HA or GA was initiated either on the day of tumor inoculation or 10 days post inoculation when palpable tumors were apparent. The drugs were administered either

topically by painting 5  $\mu$ L of a 2 mg/mL solution of the drug in dimethyl sulfoxide on the site of tumor cell inoculation or systemically by intraperitoneal injection of drug formulated in dimethyl sulfoxide.

5 Control animals received identical treatments with the appropriate vehicle alone. Animals were sacrificed 21 days after cell inoculation and well encapsulated tumors were resected and weighed. All studies involving the use of mice were carried out under protocols reviewed and approved by the  
10 Animal Care and Use Sub-committee of the National Cancer Institute.

In Vivo Anti-tumor Activity. Benzoquinonoid ansamycins as potential chemotherapeutic agents in the treatment of selected human cancers were evaluated using a  
15 tumor xenograft/nude mouse model. Preliminary experiments in athymic rats have demonstrated that intravenous infusion of 4mg/kg (or 24mg/M<sup>2</sup>) of GA is well tolerated with no overt immediate or delayed toxicity.

Table 2 depicts the effects of either topical or  
20 systemic administration of drugs to animals at the time of tumor cell inoculation. Nude mice were inoculated with tumor cells subcutaneously and therapy begun on the same day. Topical therapy consisted of applying 5  $\mu$ L of a 2 mg/mL solution of drug in dimethyl sulfoxide to the skin overlying  
25 the tumor inoculation site daily for 5 days. Systemic therapy consisted of intraperitoneal GA injection daily for 5 days at 4.5 mg/kg body weight, or about 90  $\mu$ g. Tumor formation as defined by resection of a discrete mass of 100 mg or greater was assessed 21 days after cell inoculation. A marked  
30 reduction in subsequent tumor formation was evident following both HA and GA administration. The mean weight of tumors that formed in drug-treated animals was also significantly smaller. No overt toxicity as evidenced by weight loss, decreased motor activity or local skin reaction was noted.

35 On one occasion, the tumors which formed despite topical GA treatment were resected aseptically, disaggregated in tissue culture medium and grown for 9 days *in vitro* in the presence or absence of GA. The cell cultures recovered in

this fashion grew well and, importantly, despite previous *in vivo* treatment with GA they remained fully sensitive to its cytotoxic action *in vitro*. This finding suggests that the failure of topical GA to inhibit tumor formation in all animals was a result of inadequate exposure to active drug and not acquired tumor cell resistance.

TABLE 2

*HA and GA decrease the tumorigenicity of CHP 100 in vivo*

Drug	Tumors	Topical	Tumors	Systemic
		Mean Weight (mg)		Mean Weight (mg)
DMSO Control	10/10 <sup>a</sup>	357 (49) <sup>b</sup>	10/10	436 (93)
Geldanamycin	5/10	302 <sup>c</sup> (97)	6/10	252 <sup>e</sup> (53)
Herbimycin A	7/10	221 <sup>d</sup> (49)	N.D. <sup>f</sup>	N.D.

<sup>a</sup> Number of tumors formed/number of sites inoculated.

<sup>b</sup> Numbers in parentheses refer to the standard error of the mean value.

<sup>c</sup> Comparison to control by Student's t test performed on log-transformed data  $p=0.037$

<sup>d</sup> As above,  $p=0.014$ .

<sup>e</sup> As above,  $p=0.042$ .

<sup>f</sup> N.D.=Experiment not performed.

In a similar experiment, nude mice were given a subcutaneous injection of human prostate cancer cells. These mice were treated with 5 mg/kg of HA intraperitoneally (IP). See Fig. 5. Five million human prostate cancer cells were inoculated subcutaneously. Treatment began on the day of tumor cell inoculation and was repeated every other day for a total of 3 doses. Treatment consisted of 5 mg/kg herbimycin A as an intraperitoneal injection. The animals were sacrificed and the tumors were resected and weighed two weeks after inoculation.

The ability of HA to inhibit the growth of established tumors was also examined in the nude mouse model. Initiation

of HA treatment 10 days after tumor cell inoculation at a dose of 1.5 mg/kg, given intraperitoneally every third day for 4 doses, resulted in decreased tumor mass as determined on the 21st day after tumor cell inoculation (Fig. 6). Nude mice with established subcutaneous tumors received intraperitoneal injections of either HA 1.5 mg/kg or an equivalent volume of vehicle on days 10, 13, 16 and 19 post tumor cell inoculation. Each point depicts the weight of an individual tumor mass resected on day 21. The dashed horizontal line indicates the mean weight of tumors resected from the vehicle-treated mice. Comparison of control to HA-treated tumor weights by Student's t test on log-transformed data yields  $p=0.057$ .

Because HA and GA appear to be selectively cytotoxic, it is of great interest to define their precise mechanism of action from the points of view of both basic biology as well as clinical drug development. For example, understanding the mechanism of action of these drugs may allow for identification of other sensitive tumor types. See Whitesell, et al., "Benzoquinonoid Ansamycins Possess Selective Tumoricidal Activity Unrelated to src Kinase Inhibition," *Cancer Research* 52:1721-1728 (April 1, 1992). Melanoma cell lines are being evaluated as an approach to defining the developmental/phenotypic specifically of these ansamycins.

In addition, a panel of GA derivatives was screened for potency and selectivity in tissue culture model systems. See Table 3 which lists drugs which were active against tumor development in culture. The structure-activity relationships generated should pinpoint the critical features required for activity and may suggest likely intracellular targets for these drugs.

Finally, the xenograft tumor results reported suggest promising *in vivo* anti-tumor activity for the benzoquinonoid ansamycins. The detection of any anti-tumor activity *in vivo* is encouraging given the near complete lack of baseline pharmacologic information. The antibiotics discussed here are small, lipophilic molecules that should be relatively easy to produce in bulk and, following systemic administration, should readily penetrate solid tumors and even the blood-brain

barrier. Compounds such as these may well serve as models for the development of a generation of novel drugs possessing both biologic specificity and tumoricidal pharmacology.

TABLE 3

5	17-des-O-methylgeldanamycin, geldanamycin acetate, 7',8'-benzodemethoxygeldanoxazone, hydrogeldanamycin-18,21-diacetate, 7'-(and 8')-fluorodemethoxygeldanazine,
10	7'-bromodemethoxygeldanoxazinone, 19-piperidinogeldanamycin, 8'-bromodemethoxygeldanoxazinone, 7'(or 8')-fluorodemethoxygeldanazine, and 17-amino-17-demethoxygeldanamycin.

15

The preceding description and examples are illustrative, and all references are incorporated by reference. Those skilled in the art will promptly recognize appropriate variations from the procedures as to dosing, scheduling, indications and toxicity. Thus, the invention is limited only by the scope of the appended claims.

20

WHAT IS CLAIMED IS:

1. A method for treating human malignancies selected from the group comprising primitive neuroectodermal tumors, prostate cancer, melanoma, and metastatic Ewing's sarcoma comprising administering an effective dosage of an ansamycin benzoquinone to an animal which has the malignancy.

2. The method of claim 1 wherein the administration is parenteral.

3. The method of claim 2 wherein the administration is intravenous.

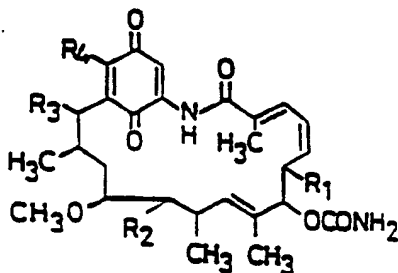
4. The method of claim 1 wherein the effective dosage is selected from a range of about 0.1 mg/kg to about 20 mg/kg.

5. The method of claim 4 wherein the effective dosage is selected from a range of about 1 mg/kg to about 10 mg/kg.

6. The method of claim 5 wherein the effective dosage is about 5 mg/kg.

7. The method of claim 1 wherein the ansamycin benzoquinone is selected from a group consisting of geldanamycin and its derivatives, herbimycin A and its derivatives, and macbecin I and its derivatives.

8. The method of claim 1 wherein the ansamycin benzoquinone has the formula



wherein  $R_1$ ,  $R_2$ ,  $R_3$  and  $R_4$  are independently selected from the group consisting of H, lower alkyl, lower alkoxy, and hydroxy.

9. The method of claim 8 wherein  $R_1$ ,  $R_2$ ,  $R_3$  and  $R_4$  are independently selected from the group consisting of H, methyl, methoxy, and hydroxy.

10. A method for treating human malignancies selected from the group comprising primitive neuroectodermal tumors, prostate cancer, melanoma, and metastatic Ewing's sarcoma comprising:

parenterally administering to a human an effective dosage of an ansamycin benzoquinone selected from the group consisting of geldanamycin and its derivatives, herbimycin A and its derivatives, and macbecin I and its derivatives, said effective dosage being an amount sufficient to ameliorate symptoms or signs of said cancer, the amount being in a range of from about 0.1 mg/kg to about 20 mg/kg.

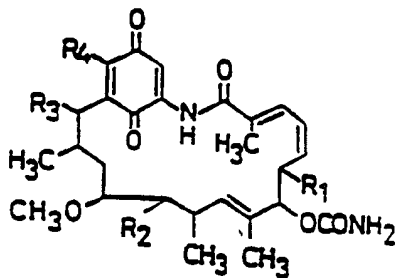
11. The method of claim 10 wherein the ansamycin benzoquinone is a geldanamycin derivative selected from the group consisting of:

17-des-O-methylgeldanamycin,  
geldanamycin acetate,  
7',8'-benzodemethoxygeldanoxazone,  
hydrogeldanamycin-18,21-diacetate,  
7'-(and 8')-fluorodemethoxygeldanazine,  
7'-bromodemethoxygeldanoxazinone,  
19-piperidinogeldanamycin,  
8'-bromodemethoxygeldanoxazinone,  
7'(or 8')-fluorodemethoxygeldanazine, and  
17-amino-17-demethoxygeldanamycin.

12. A pharmaceutical composition comprising at least one pharmaceutically acceptable excipient and an amount of an ansamycin benzoquinone effective to treat cancer in a mammal to whom at least one dose of said composition is administered wherein said cancer is selected from a group comprising

primitive neuroectodermal tumors, prostate cancer, melanoma, and metastatic Ewing's sarcoma.

13. The method of claim 12 wherein the ansamycin benzoquinone has the formula



wherein R<sub>1</sub>, R<sub>2</sub>, R<sub>3</sub> and R<sub>4</sub> are independently selected from the group consisting of H, lower alkyl, lower alkoxy, and hydroxy.

14. The method of claim 13 wherein R<sub>1</sub>, R<sub>2</sub>, R<sub>3</sub> and R<sub>4</sub> are independently selected from the group consisting of H, methyl, methoxy, and hydroxy.

15. The composition of claim 12 wherein the mammal is a human.

16. The composition of claim 12 wherein the amount of ansamycin benzoquinone is selected from a range of from about 0.1 mg/kg to about 20 mg/kg.

17. The composition of claim 16 wherein the amount of ansamycin benzoquinone is selected from a range of from about 1 mg/kg to about 15 mg/kg.

18. The composition of claim 17 wherein the amount of ansamycin benzoquinone is about 5 mg/kg.

19. The composition of claim 12 wherein the composition is adapted for parenteral administration.

20. The composition of claim 19 wherein the composition is adapted for intravenous administration.

21. The composition of claim 12 wherein the ansamycin benzoquinone is selected from a group consisting of geldanamycin and its derivatives, herbimycin A and its derivatives, and macbecin I and its derivatives.

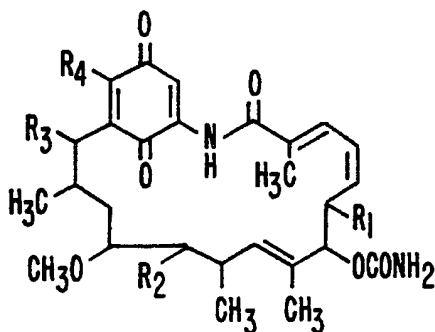
5

22. The composition of claim 12 wherein the ansamycin benzoquinone is a geldanamycin derivative selected from the group consisting of

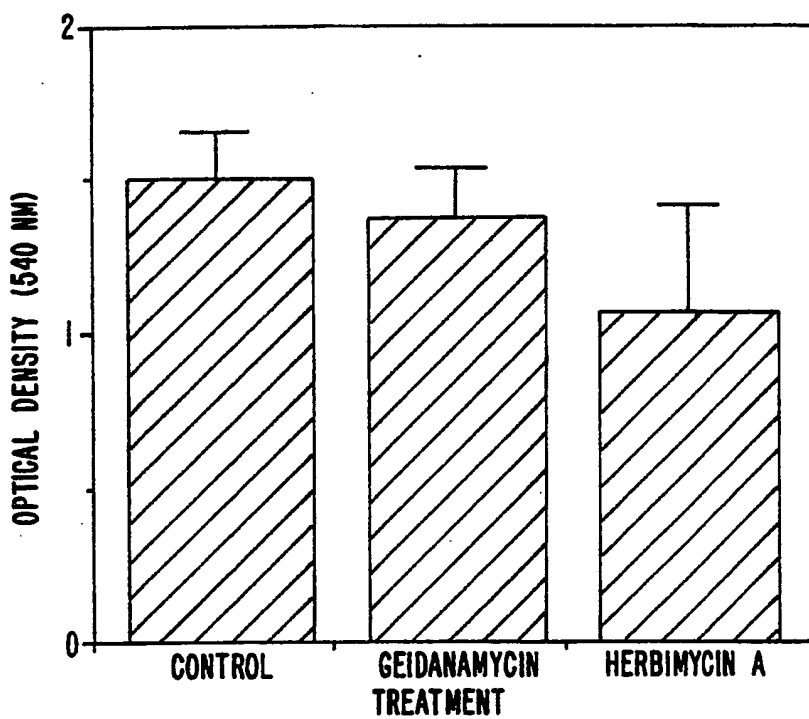
17-des-O-methylgeldanamycin,  
10 geldanamycin acetate,  
7',8'-benzodemethoxygeldanoxazone,  
hydrogeldanamycin-18,21-diacetate,  
7'-(and 8')-fluorodemethoxygeldanazine,  
7'-bromodemethoxygeldanoxazinone,  
15 19-piperidinogeldanamycin,  
8'-bromodemethoxygeldanoxazinone,  
7'(or 8')-fluorodemethoxygeldanazine, and  
17-amino-17-demethoxygeldanamycin.

20

1/5

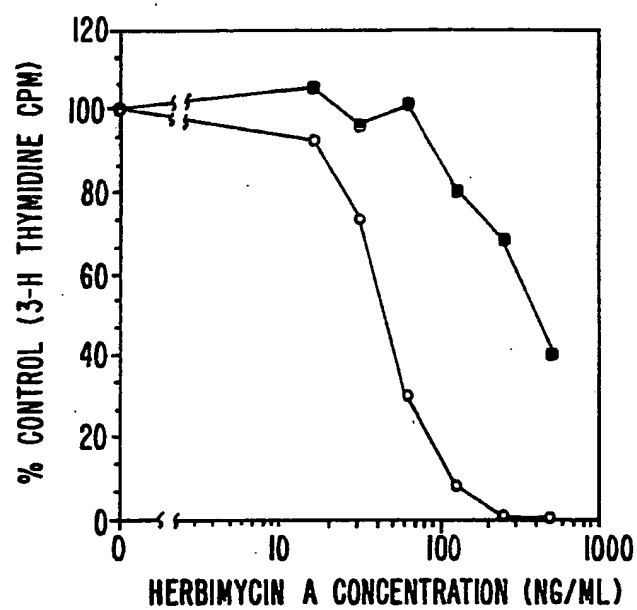
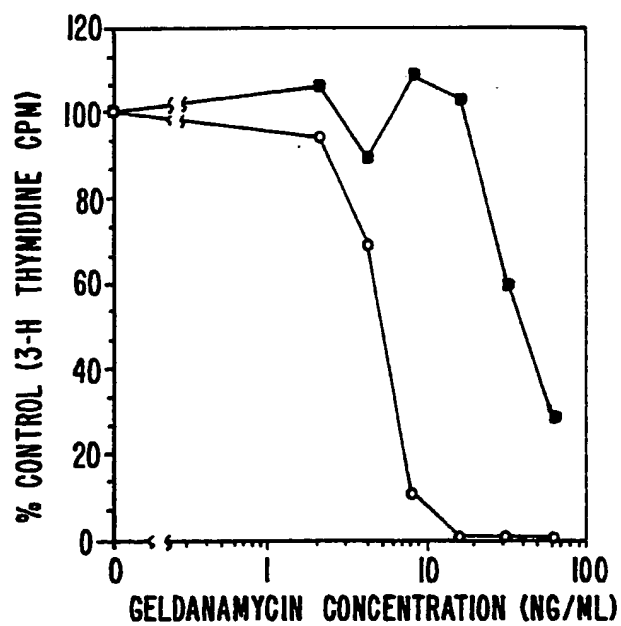


	R <sub>1</sub>	R <sub>2</sub>	R <sub>3</sub>	R <sub>4</sub>
HERBIMYCIN A	-OCH <sub>3</sub>	-OCH <sub>3</sub>	-OCH <sub>3</sub>	-H
MACBECIN I	-CH <sub>3</sub>	-OCH <sub>3</sub>	-OCH <sub>3</sub>	-H
GEIDANAMYCIN	-OCH <sub>3</sub>	-OH	-H	-OCH <sub>3</sub>

**FIG. 1.** (PRIOR ART)**FIG. 4.**

SUBSTITUTE SHEET

2/5

**FIG. 2A.****FIG. 2B.****SUBSTITUTE SHEET**

3/5

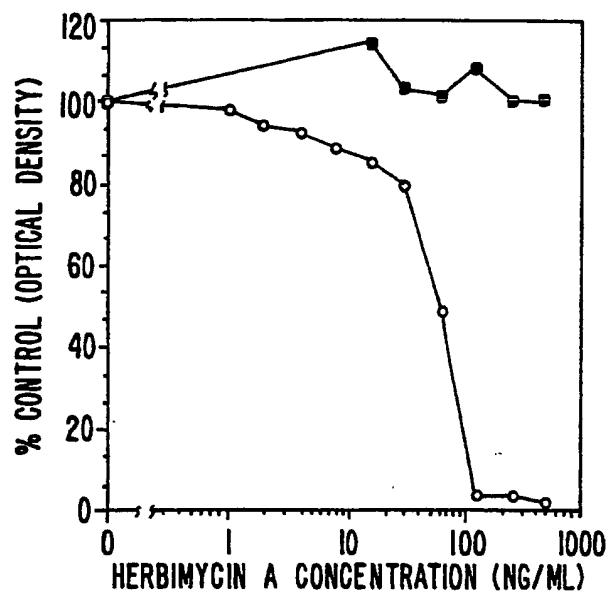


FIG. 2C.

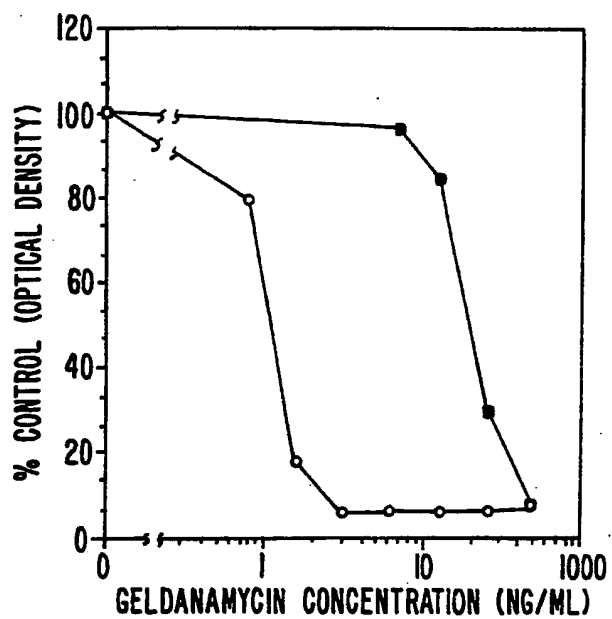
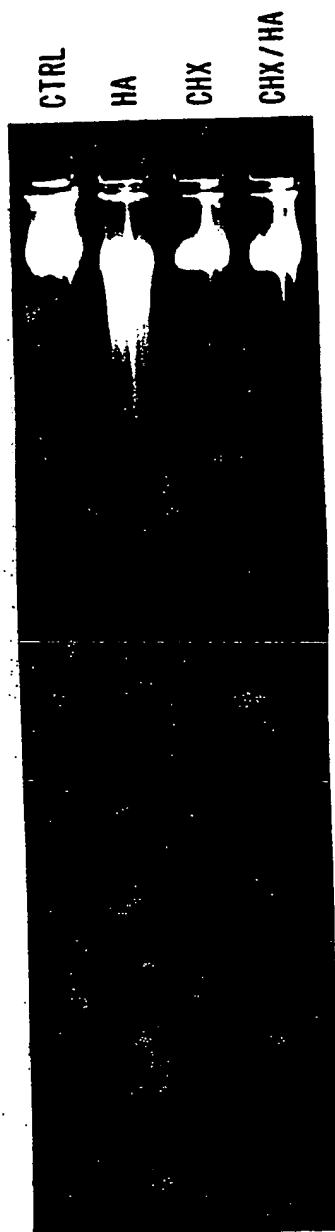


FIG. 2D.

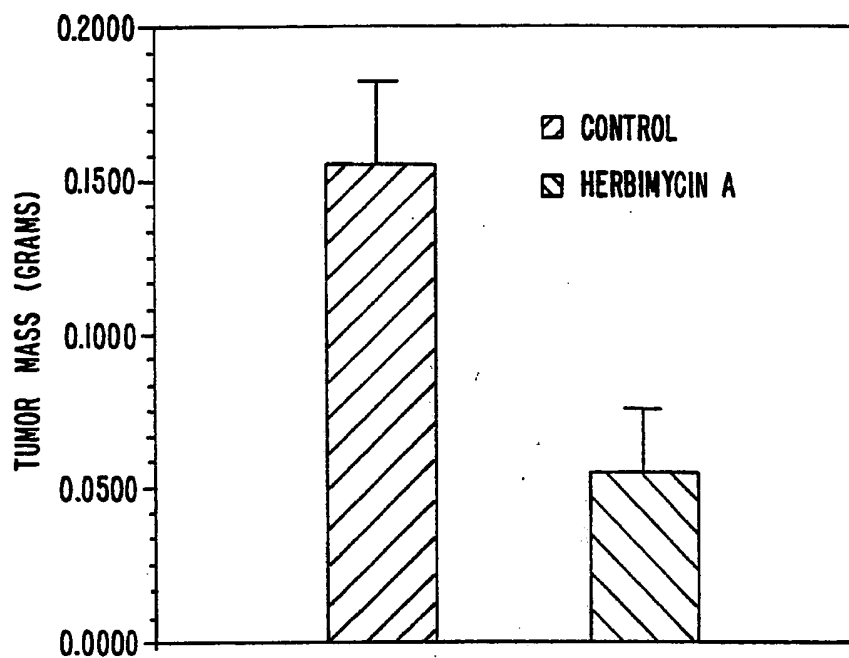
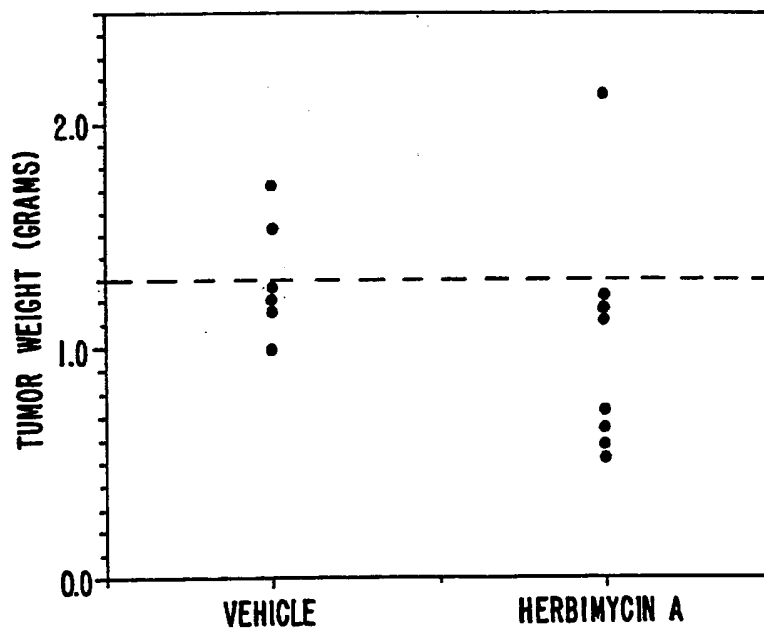
SUBSTITUTE SHEET

4/5



**FIG. 3.**

5/5

**FIG. 5.****FIG. 6.****SUBSTITUTE SHEET**

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>5</sup> :</b>  <b>A61K 31/395</b>	<b>A3</b>	<b>(11) International Publication Number:</b> <b>WO 94/08578</b>  <b>(43) International Publication Date:</b> 28 April 1994 (28.04.94)
<b>(21) International Application Number:</b> PCT/US93/09858 <b>(22) International Filing Date:</b> 14 October 1993 (14.10.93)  <b>(30) Priority data:</b> 07/961,250 14 October 1992 (14.10.92) US  <b>(71) Applicant:</b> THE GOVERNMENT OF THE UNITED STATES OF AMERICA, as represented by THE SECRETARY OF THE DEPARTMENT OF HEALTH AND HUMAN SERVICES [US/US]; Box OTT, Bethesda, MD 20892 (US).  <b>(72) Inventors:</b> WHITESELL, Luke ; 6006 Kingsford Road, Bethesda, MD 20817 (US). NECKERS, Leonard ; TREP-EL, Jane ; 5121 Wissoming Road, Bethesda, MD 20816 (US). MYERS, Charles ; 11111 Ralston Road, Rockville, MD 20852 (US).	<b>(74) Agents:</b> WEBER, Kenneth, A. et al. ; Townsend and Townsend Khourie and Crew, Steuart Street Tower, 20th floor, One Market Plaza, San Francisco, CA 94105 (US).  <b>(81) Designated States:</b> AU, CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>  <b>(88) Date of publication of the international search report:</b> 23 June 1994 (23.06.94)	
<b>(54) Title:</b> TUMORICIDAL ACTIVITY OF BENZOQUINONOID ANSAMYCINS AGAINST PROSTATE CANCER AND PRIMITIVE NEURAL MALIGNANCIES  <b>(57) Abstract</b>  The invention provides methods and compositions for treating selected human malignancies including use of certain ansamycin benzoquinones.		

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	GB	United Kingdom	MR	Mauritania
AU	Australia	GE	Georgia	MW	Malawi
BB	Barbados	GN	Guinea	NE	Niger
BE	Belgium	GR	Greece	NL	Netherlands
BF	Burkina Faso	HU	Hungary	NO	Norway
BG	Bulgaria	IE	Ireland	NZ	New Zealand
BJ	Benin	IT	Italy	PL	Poland
BR	Brazil	JP	Japan	PT	Portugal
BY	Belarus	KE	Kenya	RO	Romania
CA	Canada	KG	Kyrgyzstan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LU	Luxembourg	TD	Chad
CS	Czechoslovakia	LV	Latvia	TC	Togo
CZ	Czech Republic	MC	Monaco	TJ	Tajikistan
DE	Germany	MD	Republic of Moldova	TT	Trinidad and Tobago
DK	Denmark	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	US	United States of America
FI	Finland	MN	Mongolia	UZ	Uzbekistan
FR	France			VN	Viet Nam
GA	Gabon				

## INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 93/09858

A. CLASSIFICATION OF SUBJECT MATTER  
IPC 5 A61K31/395

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 5 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CANCER RES., vol.52, no.7, 1 April 1992 pages 1721 - 1728 L.WHITESSELL ET AL. 'Benzoquinonoid Ansamycins Possess Selective Tumoricidal Activity Unrelated to src Kinase Inhibition' cited in the application see the whole document ---	1-22
X	NIPPON GEKA HOKAN (ARCH. JPN. CHIR.), vol.60, no.2, 1 March 1991 pages 113 - 121 M. MATSUMOTO 'Inhibitors for Protein Tyrosine Kinases, Erbstatin, Genistein and Hernimycin A, Induce Differentiation of Human Neural Tumor Cell Lines' see abstract --- -/--	1-22

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

## \* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

15 December 1993

Date of mailing of the international search report

20.04.94

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,  
Fax (+31-70) 340-3016

Authorized officer

THEUNS, H

## INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 93/09858

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CANCER RES., vol.48, no.22, 15 November 1988 pages 6530 - 6534 P.N.PREIS ET AL. 'Neuronal Cell Differentiation of Human Neuroblastoma Cells by Retinoic Acid plus Herbimycin A' see abstract ----	1-22
X	GB,A,2 042 523 (KAKEN CHEMICAL CO., LTD.) 24 September 1980 see the whole document ----	12,15-22
X	DE,A,32 34 203 (TAKEDA CHEMICAL INDUSTRIES, LTD.) 7 July 1983 see the whole document -----	12,15-22

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 93/09858

**Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:  
REMARK: Although claims 1-11 and 13,14 are directed to a method of treatment of the human/animal body, the search has been based on the alleged effects of the compound/composition.
2. ☒ Claims Nos.: 7,10,13,14,21.  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:  
Claim 13 refers to claim 12; however, there is no method in claim 12.  
The expression "derivatives" is not sufficient for chemical characterisation of compounds (claim 21)
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

For further information please see Form PCT/ISA/206 sent 20 01 94.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

## INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 93/09858

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
GB-A-2042523	24-09-80	JP-C- 1490629	07-04-89
		JP-A- 55111469	28-08-80
		JP-B- 63034866	12-07-88
		JP-C- 1464605	10-11-88
		JP-A- 55111470	28-08-80
		JP-B- 63012065	17-03-88
		JP-C- 1417696	22-12-87
		JP-A- 55111485	28-08-80
		JP-B- 62019430	28-04-87
		JP-C- 1464607	10-11-88
		JP-A- 55113767	02-09-80
		JP-B- 63012066	17-03-88
		AU-B- 532333	29-09-83
		AU-A- 5443080	28-08-80
		CA-A- 1113935	08-12-81
		DE-A, C 3006097	28-08-80
		FR-A, B 2449084	12-09-80
		NL-A- 8000857	21-08-80
		US-A- 4261989	14-04-81
DE-A-3234203	07-07-83	JP-C- 1707149	27-10-92
		JP-B- 3072618	19-11-91
		JP-A- 58212793	10-12-83
		JP-C- 1650614	30-03-92
		JP-B- 3015633	01-03-91
		JP-A- 58074651	06-05-83
		JP-C- 1671564	12-06-92
		JP-B- 3037559	05-06-91
		JP-A- 58076093	09-05-83
		FR-A, B 2512819	18-03-83
		GB-A, B 2106111	07-04-83
		US-A- 4421687	20-12-83
		US-A- 4512975	23-04-85
		CA-A- 1185550	16-04-85
		US-A- 4421688	20-12-83